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ORIGINAL ARTICLE

DNA barcoding reveals long-term speciation processes in subspecies of the *Melipona* (*Michmelia*) *seminigra* complex (Hymenoptera: Apidae)

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Key words. mtDNA, COI diversity, genetic distance, barcoding delimitation, bees, Meliponini

Abstract. The stingless bee *Melipona* (*Michmelia*) *seminigra* Friese is a polytypic species widely distributed in Brazilian Amazon and Bolivia. Seven subspecies are recognized, four are described, which inhabit mutually exclusive areas in the Amazon basin, although zones of hybridization are recorded. The three other subspecies, despite being recognized by taxonomists are undescribed. *Melipona seminigra* is a good honey-producer and an important pollinator of native flora and crops. Partial DNA sequence of cytochrome c oxidase subunit 1 (COI) mitochondrial gene (526 bp) was used to identify the four described subspecies of *M. seminigra* (*M. s. abunensis*, *M. s. merrillae*, *M. s. pernigra*, *M. s. seminigra*) and two other possible subspecies (*M. s.* ssp1 and *M. s.* ssp2). We added public data (sequences of ten other *Melipona* species) of the same subgenera and carried out phylogenetic analyses. The aim was to evaluate if subspecies in the *M. seminigra* complex could be delimited using COI and measure the genetic distances between them. Our results revealed that the genetic distances between subspecies of *M. seminigra* ranged from 0.4 to 2.7% (average 1.80 ± 0.47) and among *Melipona* species from 0.2 to 2.9% (average 2.13 ± 0.5). The average haplotype diversity was 0.8770 ± 0.0140 and average nucleotide diversity 0.0166 ± 0.0004. Phylogenetic and clustering analysis revealed well delimited clusters for subspecies of *M. seminigra* and that the inter subspecies divergences are similar to inter species divergence. Our findings indicate that the COI gene can be used for delimiting subspecies of *M. seminigra*.

INTRODUCTION

Stingless bees (Hymenoptera: Apidae) occur in tropical and subtropical regions of the world with the centre of diversity in the Neotropics (Michener, 2007). As they occur in forests, they play a significant role as pollinators in Amazonian ecosystems (Roubik, 1989; Carvalho-Zilse & Nunes-Silva, 2012). They also pollinate crops (Heard, 1999) and are suitable for pollinating plants in greenhouses (Slaa et al., 2006). Among the stingless bees, *Melipona* Illiger 1806, is the most species-rich genus (Camargo & Pedro, 2013) and are distributed from Mexico to Argentina (Michener, 2007) with the greatest diversification and species richness in the Amazon Basin (Silveira et al., 2002; Camargo & Pedro, 2013).

Melipona (*Michmelia*) *seminigra* Friese, 1903 is a polytypic species that occurs in Bolivia (El Beni Department) and Brazil (Acre, Amazonas, Maranhão, Mato Grosso, Pará, Rondônia, Roraima and Tocantins States) (Pedro, 2014). The role of *M. seminigra* as a pollinator and seed disperser in Amazonian ecosystems and plants cultivated by natives

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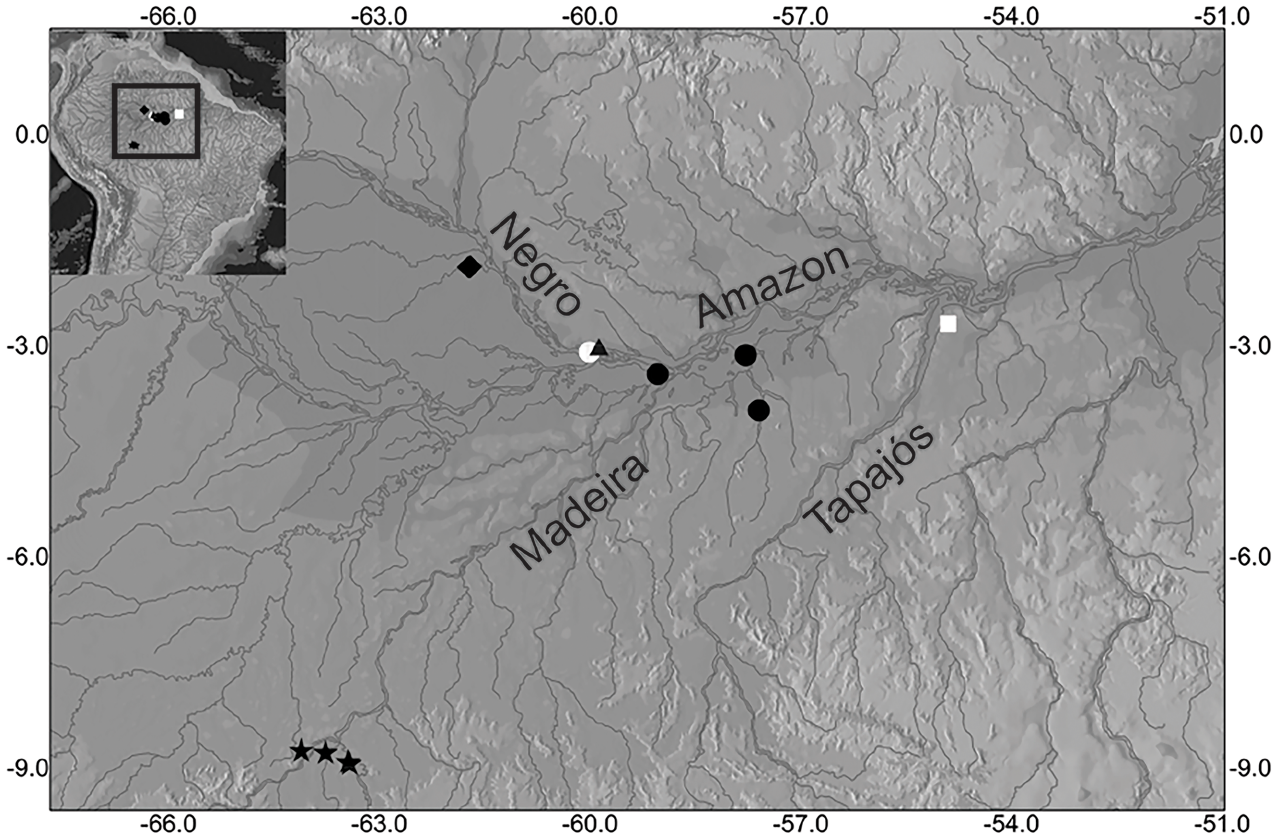


Fig. 1. Sites sampled in the Brazilian Amazon. Black star – *Melipona seminigra abunensis* (Porto Velho, Rondônia); white circle – *M. s. merrillae* (Grupo de Pesquisas em Abelhas (GPA) of the Instituto Nacional de Pesquisas da Amazônia (INPA); black triangle – *M. s.* ssp1 (Puraquequara, Manaus, Amazonas); white square – *M. s. pernigra* (Belterra, Pará); black circle – *M. s. seminigra* (Boa Vista do Ramos and Maués, Amazonas); black square – *M. s.* ssp2 (Parque Nacional do Jaú, Amazonas). Coordinates of the sites sampled are in Table 1.

is well documented (Absy & Kerr, 1977; Bacellar-Lima et al., 2006). It is a good honey and pollen producer and only mildly aggressive, being the most reared stingless bee by Brazilian Amazon people, both as an economic alternative and for conservation purposes (Carvalho-Zilse & Nunes-Silva, 2012). At present seven subspecies of *M. seminigra* are recognized, four of them are described: *M. s. abunensis* Cockerell, 1912, *M. s. merrillae* Cockerell, 1919, *M. s. pernigra* Moure & Kerr, 1950 and *M. s. seminigra* Friese, 1903; and three others are undescribed (Camargo & Pedro, 2013). They inhabit mutually exclusive geographic areas, but hybridization zones are reported in some cases (Camargo & Pedro, 2013).

We aimed to evaluate if the cytochrome oxidase subunit 1 (COI) gene (Hebert et al., 2003a, b) could be used to determine the boundaries of the subspecies of *M. seminigra*. We hypothesized that reproductive isolation in allopatry led to morphological differences in the *M. seminigra* subspecies complex (Moure & Kerr, 1950) and also resulted in genetic differentiation such that the COI gene could be used to delimit them. Our understanding is that DNA-based subspecies identification of *M. seminigra* could be a valuable tool for future studies on the evolution of this group in the Amazon basin and guide the breeding of *M. seminigra* in the Meliponiculture activity of local people.

2. MATERIAL AND METHODS

2.1. Bee sampling

We sampled thirty-one colonies of *Melipona (M.) seminigra*, both natural and manipulated, in the Brazilian Amazon. Sampling sites (Fig. 1) were chosen based on the distribution of *M. seminigra* in the Amazon basin, mainly the specific places associated with the described subspecies (Camargo & Pedro, 2013). All the described subspecies were sampled (Fig. 2a, b, c, d) plus two putative subspecies (Fig. 2e–f), which are not described but likely to differ from the undescribed subspecies recognized by Camargo & Pedro (2013). The coordinates of the sites, the number of colonies sampled per subspecies, number of individuals sequenced per colony and type of colony (whether natural or manipulated) are listed in Table 1. Although *M. seminigra* colonies are theoretically monogynous, more than one individual from both types of colonies were sequenced to confirm monogyny in both types of colonies.

All samples were preserved in absolute ethanol and stored at –20°C. The four recognized subspecies were identified based on the morphological characters described by Moure & Kerr (1950) and by comparison with specimens in the entomological collection of the Instituto Nacional de Pesquisas da Amazônia (INPA). Sampling data, voucher specimens, remains of specimens and GenBank access numbers of all sequenced specimens are described in Table S1. Voucher specimens and remains are deposited in the Grupo de Pesquisas em Abelhas (GPA) of INPA.

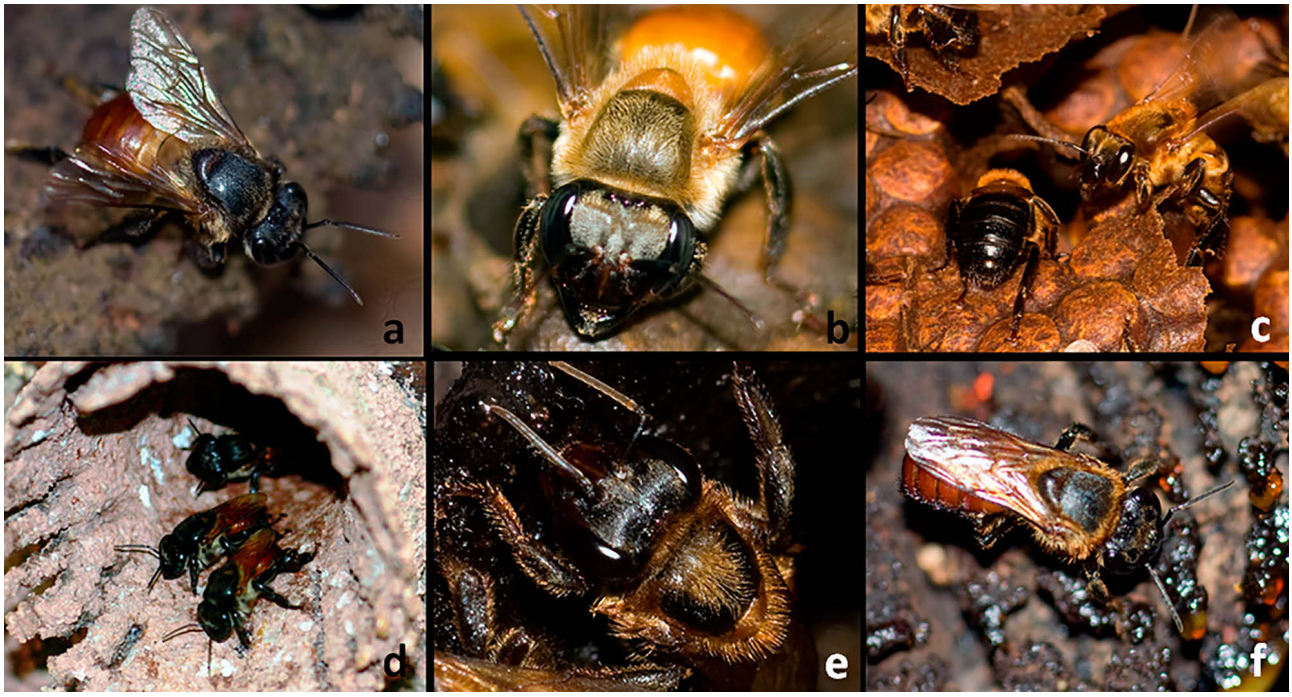


Fig. 2. The barcoded subspecies of *Melipona seminigra*: a – *M. s. abunensis*; b – *M. s. merrillae*; c – *M. s. pernigra*; d – *M. s. seminigra*; e – *M. s*. ssp1; f – *M. s*. ssp2.

Table 1. Sampling data of *Melipona seminigra* subspecies carried out for this study: *M. s. abunensis*, seven colonies sampled at Rondônia (RO); *M. s. merrillae*, three colonies sampled at Manaus, Amazonas (AM); *M. s. pernigra*, four colonies sampled at Belterra, Pará (PA); *M. s. seminigra*, four colonies sampled at Boa Vista do Ramos (AM) and three colonies sampled at Maués (AM); *M. s*. ssp1 (new putative subspecies), three colonies sampled at Puraquequara, Manaus (AM); *M. s.* ssp2 (putative subspecies, not described morphologically), seven colonies sampled at Parque Nacional do Jaú (AM). N – individuals sequenced per colony; * – natural colony; ** – manipulated colony; LM – left margin; GPA – Grupo de Pesquisas em Abelhas; INPA – Instituto Nacional de Pesquisas da Amazônia.

Subspecies	Colony	N	Sites sampled	Coordinates
M. s. abunensis	1B*	2	Sta Marcelina, BR 364 km 17, RO, Brazil	8°47'33.4''S, 63°44'21.4''W
M. s. abunensis	1C*	2	Sta Marcelina, BR 364 km 17, RO, Brazil	8°47'33.4''S, 63°44'21.4''W
M. s. abunensis	1D*	3	Sta Marcelina, BR 364 km 17, RO, Brazil	8°47'33.4''S, 63°44'21.4''W
M. s. abunensis	1E*	2	Cadeias of Jamari, BR 364 km 62, RO, Brazil	8°55'22.6''S, 63°25'17.4''W
M. s. abunensis	1F*	2	Cadeias of Jamari, BR 364 km 67, RO, Brazil	8°56'55.4''S, 63°23'45.1''W
M. s. abunensis	1G*	1	Igarapé Jatuarana, Madeira River (LM), RO, Brazil	8°45'48.1''S, 64°05'09.0''W
M. s. abunensis	1H*	4	Igarapé Jatuarana, Madeira River (LM), RO, Brazil	8°45'48.1''S, 64°05'09.0''W
M. s. merrillae	2Ca**	4	GPA/INPA, Manaus, AM, Brazil	3°05'83.8''S, 59°59'10.3''W
M. s. merrillae	2Ka**	2	GPA/INPA, Manaus, AM, Brazil	3°05'83.8''S, 59°59'10.3''W
M. s. merrillae	2Pa**	2	GPA/INPA, Manaus, AM, Brazil	3°05'83.8''S, 59°59'10.3''W
M. s. ssp1	2T*	4	Puraquequara, Manaus, AM, Brazil	3°05'95.6''S, 59°84'68.6''W
M. s. ssp1	2U*	3	Puraquequara, Manaus, AM, Brazil	3°05'95.6''S, 59°84'68.6''W
M. s. ssp1	2V*	2	Puraquequara, Manaus, AM, Brazil	3°05'95.6''S, 59°84'68.6''W
M. s. pernigra	3Aa**	2	Belterra, PA, Brazil	2°68'29.3''S, 54°93'25.3''W
M. s. pernigra	3Ab**	2	Belterra, PA, Brazil	2°68'29.3''S, 54°93'25.3''W
M. s. pernigra	3Ba**	2	Belterra, PA, Brazil	2°68'29.3''S, 54°93'25.3''W
M. s. pernigra	3Bb**	2	Belterra, PA, Brazil	2°68'29.3''S, 54°93'25.3''W
M. s. pernigra	3Ca**	4	Belterra, PA, Brazil	2°68'29.3''S, 54°93'25.3''W
M. s. pernigra	3Cb**	2	Belterra, PA, Brazil	2°68'29.3''S, 54°93'25.3''W
M. s. pernigra	3Da**	1	Belterra, PA, Brazil	2°68'29.3''S, 54°93'25.3''W
M. s. seminigra	4A**	1	Boa Vista do Ramos, AM, Brazil	3°08'24.9''S, 57°45'31.3''W
M. s. seminigra	4B**	3	Boa Vista do Ramos, AM, Brazil	3°08'24.9''S, 57°45'31.3''W
M. s. seminigra	4D**	2	Boa Vista do Ramos, AM, Brazil	3°08'24.9''S, 57°45'31.3''W
M. s. seminigra	4E**	1	Boa Vista do Ramos, AM, Brazil	3°55'27.9''S, 57°34'18.5''W
M. s. seminigra	4F**	1	Maués, AM, Brazil	3°24'35.8''S, 57°41'01.3''W
M. s. seminigra	4G**	4	Maués, AM, Brazil	3°24'35.8''S, 57°41'01.3''W
M. s. seminigra	4H**	2	Maués, AM, Brazil	3°24'35.8''S, 57°41'01.3''W
M. s. ssp2	5A*	2	National Park of Jau, AM, Brazil	1°53'15.5''S, 61°41'20.6''W
M. s. ssp2	5B*	2	National Park of Jau, AM, Brazil	1°53'20.6''S, 61°41'46.0''W
M. s. ssp2	5C*	2	National Park of Jau AM, Brazil	1°52'46.0''S, 61°40'27.0''W
M. s. ssp2	5D*	2	National Park of Jau AM, Brazil	1°53'29.2''S, 61°41'02.5''W
M. s. ssp2	5E**	2	National Park of Jau AM, Brazil	1°58'12.7''S, 61°35'20.0''W
M. s. ssp2	5F**	1	National Park of Jau AM, Brazil	1°58'12.7''S, 61°35'20.0''W
M. s. ssp2	5H**	1	National Park of Jau AM, Brazil	1°58'12.7''S, 61°35'20.0''W
Total		74		

2.2. DNA analysis and sequencing

Genomic DNA was extracted from thorax tissue of workers using the Genomic DNA Purification Kit (Promega) based on the manufacturer's protocol and quantified by comparison with lambda phage DNA in 1% agarose gels. The partial cytochrome c oxidase subunit 1 (COI) gene of seventy-four workers of described subspecies of *M. seminigra* was sequenced: *M. s. abunensis*, *M. s. merrillae*, *M. s. pernigra* and *M. s. seminigra*, plus two putative subspecies: *M. s.* ssp1 and *M. s*. ssp2, using the primer pair CI-F 1632 (5'TGTCAAATTTATAAT3') and CI-R-2191 (5'GGTAAAATTAAAATATAAACTTC3') (Kambhampati & Smith, 1995) modified by Simon et al. (1994). Polymerase chain reactions (PCRs) were carried out in a total volume of 25 µL with 2.0 µL 50 ng DNA template, 2.5 µL 10×buffer, 2.0 µL 50 mM $MgCl_2$, 5.0 µL 1 mM DNTP's, 2.0 µL 2 mM forward primer, 2.0 µL 2 mM reverse primer, 0.2 µL (5 U/µL) *Taq* DNA polymerase and 9.3 µL ddH_2O. PCR cycling was done with initial denaturation for two min at 95°C followed by 30 cycles of 30 s at 95°C, 1 min at 45°C, 1 min at 72°C and 10 min at 72°C for final extension. PCR products were run on 1% agarose gels and purified using polyethylene glycol 8000/ethanol precipitation (Paithankar & Prasad, 1991). Nucleotide sequencing was carried out in both directions according to the cycling protocol of Platt et al. (2007) using a genetic analyser (ABI 3130XL sequencer, ThermoFisher) based on the manufacturer's protocol.

2.3. Data analysis: Sequence evaluation and cluster delimitation

Partial consensus sequences of COI were edited using the program SeqScape 2.7 (Applied Biosystems). After terminal end elimination, we obtained a final matrix with 526 aligned bp (base pairs). Ten additional public sequences of *Melipona* species of the *Michmelia* subgenus were included: EU163125, *M. fulva*; EU163144, *M. lateralis*; EU163136, *M. fuscopilosa*; EU163159, *M.* sp. *eburnea* group MP85; EU163121, *M.* aff. *costaricaensis* MP21; EU163096, *M. panamica* MP1; EU163113, *M. melanopleura* MP12; EU16313, *M. rufiventris* MP24; EU163152, *M. scutellaris* MP 78, EU163138, "*M. seminigra atrofulva*" MP38) (Ramírez et al., 2010). As an outgroup we used *Melipona captiosa* (GenBank access EU162976), which was also sequenced by Ramirez et al. (2010). The sequence alignment was done using ClustalW (Thompson et al., 1994) in BioEdit (Hall, 1999) and compared using the Neighbour-Joining (NJ) method corrected by Kimura 2-parameter (K2P) (Kimura, 1980) implemented in MEGA 5 (Tamura et al., 2011). Two matrices were used for estimating the phylogeny – one with all species and subspecies, the other with the subspecies only and one outgroup. We carried out phylogenetic reconstruction using maximum parsimony (MP) implemented in PAUP 4.0 (Swofford, 2002) and Maximum Likelihood (ML) estimation using the RAxML web servers (Stamatakis et al., 2008). The model of molecular evolution selected was GTR+I+G using the program MODELTEST version 3.7 (Posada & Crandall, 1998) according to Akaike Information Criterion (AIC). For a tree topology with the smallest number of character changes, we used a heuristic search with tree bisection and reconnection (TBR). Tree node supports were obtained based on 1,000 nonparametric bootstrap replicates. We tested for admixture among subspecies using a clustering analysis implemented in BAPS5 (Bayesian Analysis of Population Structure) (Corander & Tang, 2007; Corander et al., 2008) and our subspecies matrix. First, we ran a mixture analysis that identified the number of clusters in our dataset. This analysis was run several times to confirm the results converge. Then, we used the mixture results to carry out an admixture analysis using 100 replicates, 200 reference individuals and 10 iterations per individual. We used MEGA 5 to do the calculations of the genetic distances among subspecies. To assess haplotype richness, nucleotide diversity and genetic variability we used software DNAsp v 5.0 (Librado & Rozas, 2009).

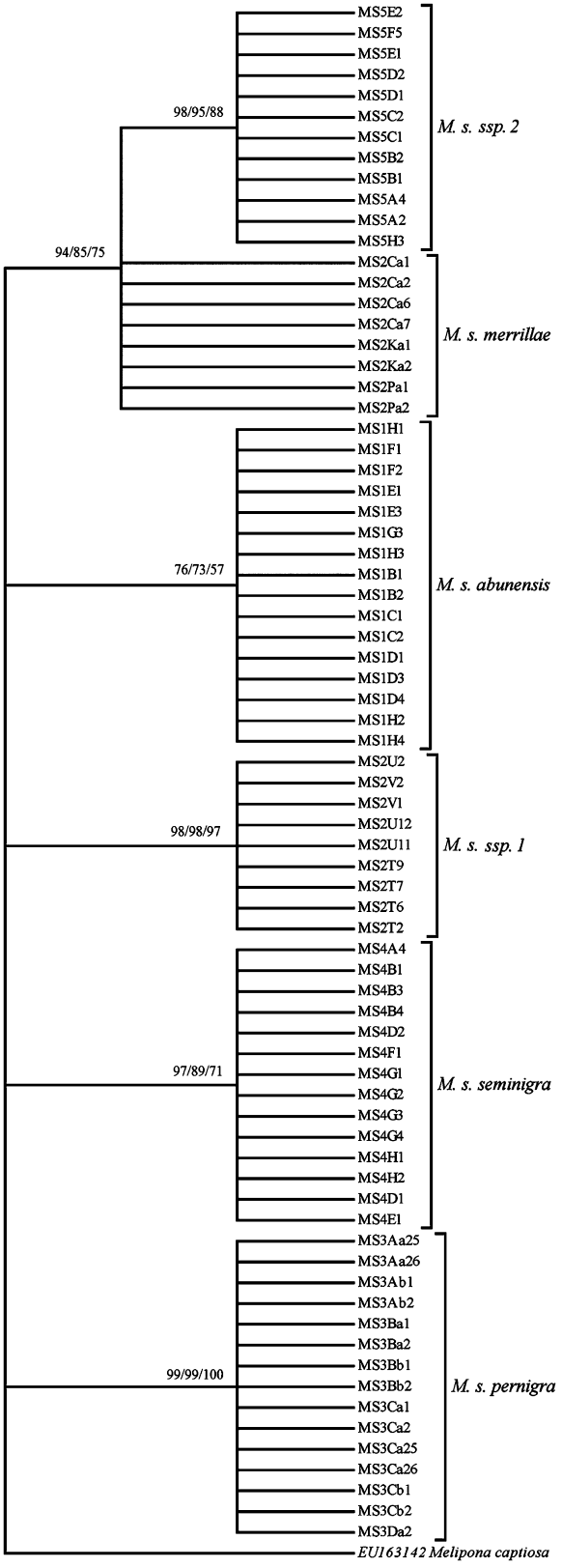


Fig. 3. Dendrogram based on the Neighbour-Joining tree for subspecies of *Melipona seminigra*, corrected using Kimura 2-parameter (K2P) (Kimura, 1980) implemented in MEGA 5 (Tamura et al., 2011).

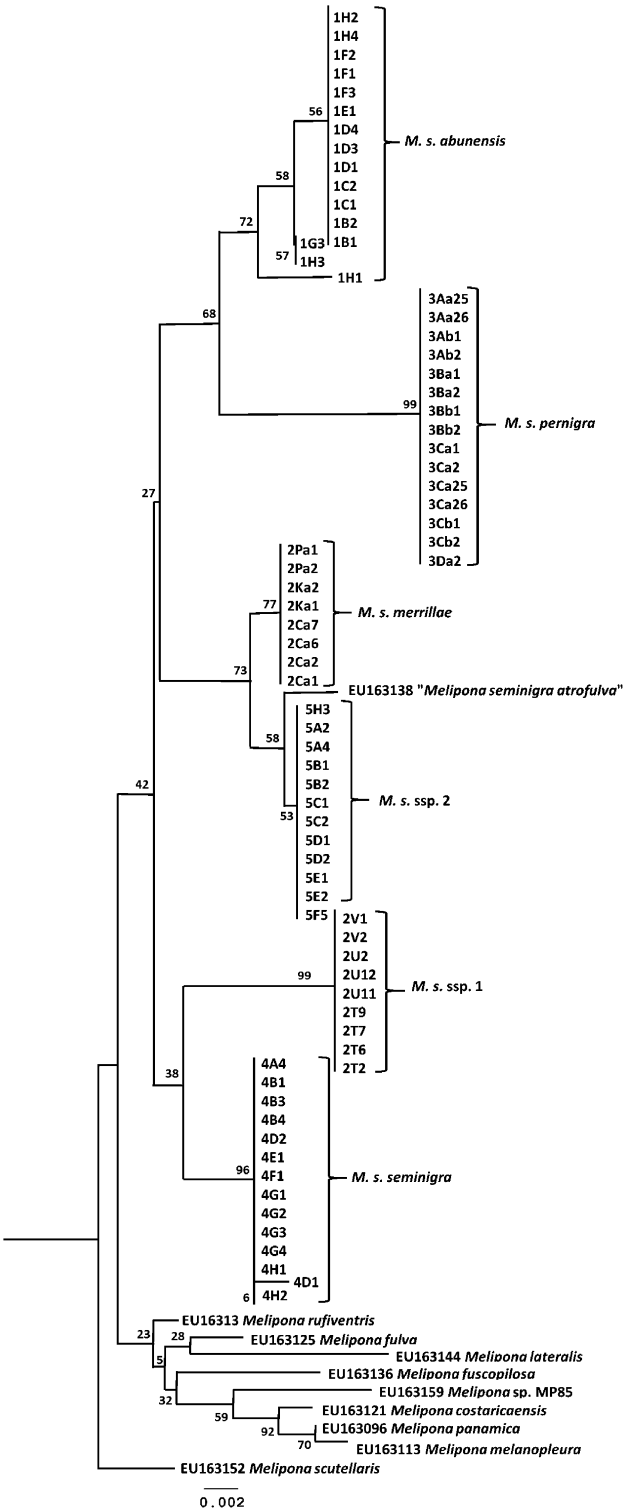


Fig. 4. Phylogeny obtained using the species matrix, which includes the subspecies of the *Melipona seminigra* complex and one outgroup. Phylogenetic reconstruction used Maximum Parsimony (MP) implemented in PAUP 4.0 (Swofford, 2002) and Maximum Likelihood (ML) estimation using the RAxML web servers (Stamatakis et al., 2008).

3. RESULTS

The phylogeny based on distance estimates for both the *Melipona seminigra* subspecies complex (Fig. 3) and all *Melipona* species (Fig. 4) revealed that the COI gene fragment identified the boundaries of the subspecies in the *M. seminigra* complex, which was also confirmed by the results of the admixture analysis (Fig. 5). The observed genetic divergence among subspecies of *M. seminigra* ranged from 0.4 to 2.7% (average 1.8 ± 0.47) and the divergence among species ranged from 0.2 to 2.9% (average 2.13 ± 0.50) (Tables 2 and 3). The average divergence values between subspecies of *M. seminigra* (Table 3) are close to the average divergence values for species of *Melipona* herein analysed (EU163125, *M. fulva*; EU163144, *M. lateralis*; EU163136, *M. fuscopilosa*; EU163159, *M.* sp. *eburnea* group MP85; EU163121, *M.* aff. *costaricaensis* MP21; EU163096, *M. panamica* MP1; EU163113, *M. melanopleura* MP12; EU16313, *M. rufiventris* MP24; EU163152, *M. scutellaris* MP 78, EU163138, "*M. seminigra atrofulva*" MP38 (Ramírez et al., 2010) (Fig. 6, average values in Table 2). The highest genetic divergence among subspecies was recorded for *M. s. pernigra* (Table 3). In the full matrix containing 526 bp of the *M. seminigra* complex, there were 28 variable sites and 30 informative mutations. The average nucleotide composition recorded was 43.7% T, 33.1% A, 13.5% C and 9.7% G, resulting in a G + C content of 23%. We recorded a total of 13 haplotypes, resulting in an overall haplotype diversity of 0.8770 ± 0.0140 and a nucleotide diversity of 0.0166 ± 0.0004. The sequencing results confirmed monogyny both in natural and manipulated colonies. Sequences were deposited in GenBank under the accession numbers KF113947–KF114020 (Table S1). There were six well delimited clusters for the subspecies of *Melipona seminigra* and the genetic divergence among them can be easily accessed using the number of base substitutions per site (Fig. 7).

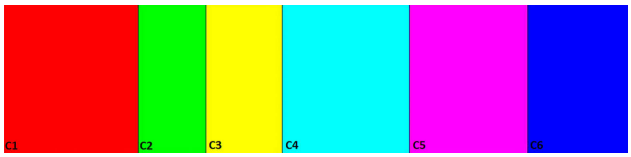


Fig. 5. BAPS5 clustering analysis of the subspecies of the *Melipona seminigra* complex (based on 100 replicates, 200 reference individuals and 10 iterations per individual) showing six well defined clusters: C1 – *M. s. abunensis*; C2 – *M. s. merrillae*; C3 – *M. s.* ssp1; C4 – *M. s. pernigra*; C5 – *M. s. seminigra*; C6 – *M. s.* ssp2.

4. DISCUSSION AND CONCLUSION

The identification of subspecies of *Melipona seminigra* using the cytochrome c oxidase subunit 1 (COI) gene is reliable as evidenced by phylogenetic (Figs 3 and 4) and clustering analyses (Fig. 5) (Corander & Tang, 2007; Corander et al., 2008). The COI gene is an efficient tool for species identification in various groups of animals (Hebert et al., 2003a, b, 2004; Hajibabaei et al., 2006, 2007; Waugh, 2007). The delimitation of animal subspecies using the COI gene is uncommon, however, it has been successfully used to delimit honeybee subspecies (Franck et al., 2001; Alattal et al., 2014; Syromyatnikov et al., 2018; Ilyasov et al., 2019; Alghamdi & Alattal, 2021). Our findings for *M. seminigra* indicate that the use of the COI gene to delimit

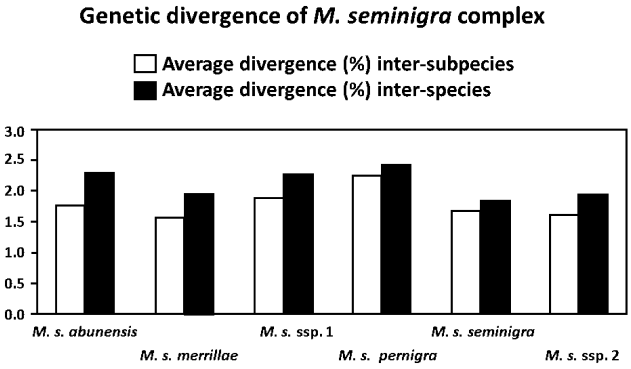
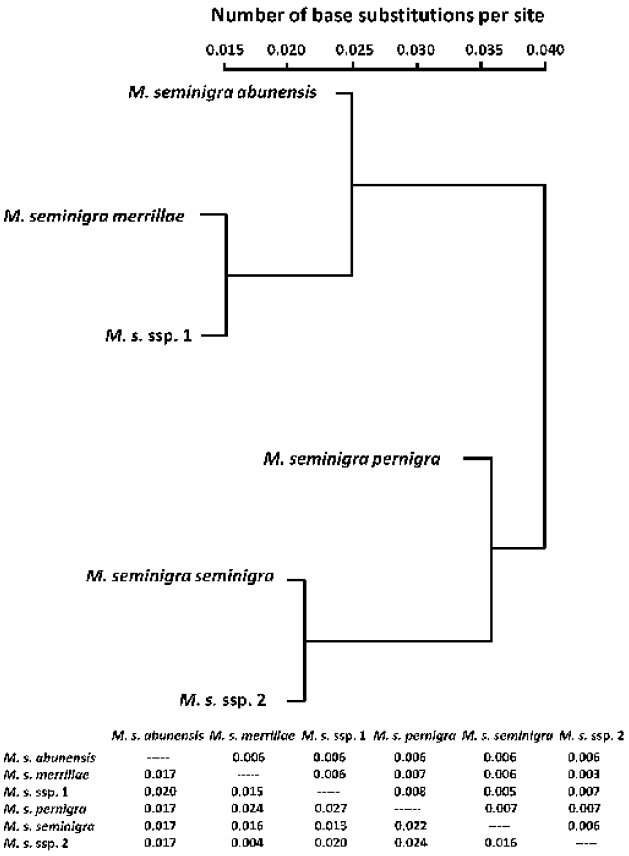


Fig. 6. The average divergence value for subspecies of *Melipona seminigra* (Table 3) is close to the average divergence value for the species of *Melipona* analysed (average values in Table 2).



	M. s. abunensis	*M. s. merrillae*	*M. s. ssp. 1*	*M. s. pernigra*	*M. s. seminigra*	*M. s. ssp. 2*
M. s. abunensis	-----	0.006	0.006	0.006	0.006	0.006
M. s. merrillae	0.017	-----	0.006	0.007	0.006	0.003
M. s. ssp. 1	0.020	0.015	-----	0.008	0.005	0.007
M. s. pernigra	0.017	0.024	0.027	-----	0.007	0.007
M. s. seminigra	0.017	0.016	0.013	0.022	----	0.006
M. s. ssp. 2	0.017	0.004	0.020	0.024	0.016	----

Fig. 7. Estimates of evolutionary divergence between sequence pairs in subspecies of *Melipona seminigra* showing the number of base substitutions. Standard error estimate(s) are shown above the matrix diagonal. Analyses were done using the Kimura 2-parameter model (Kimura, 1980) and included 74 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Dendrogram was clustered using complete linkage.

subspecies of bees seems to be promising, as previously noted by Ilyasov et al. (2019). The COI genetic divergence threshold varies according to the taxonomic level and animal group (Avise & Aquadro, 1982). Inter species divergences of 3% are reported for Lepidoptera, other than congeneric species, which have a genetic divergence between 0.6 and 2.0% due to their more recent origin (Hajibabaei et al., 2006). An average of 1.3 ± 0.7% divergence is reported for lineages of *Apis mellifera*, which indicates long–term geographic isolation (Garnery et al., 1992). In stingless bees of the genus *Scaptotrigona* a mean of 2.79% and 0.7% genetic divergence inter- and intra- specifically, respectively, are reported (Hurtado-Burillo et al., 2013). For the species of *Melipona*, herein analysed, we recorded genetic divergences ranging from 0.2 to 2.9% (average 2.13 ± 0.50) (Table 2) whereas the genetic divergences between subspecies in the *Melipona seminigra* complex ranged from 0.4% (between *M. s. merrillae* and *M. s.* ssp2) to 2.7% (between *M. s. pernigra* and *M. s.* ssp1), with an average of 1.80 ± 0.47 (Table 3). Therefore, the genetic divergence among subspecies of *Melipona seminigra* is close to that among the species of *Melipona* belonging to the same subgenus, herein barcoded (Fig. 6). The subspecies taxonomic category has been a subject of discussion since the 19th century (Mayr, 1942, 1963; Winker, 2010). It is formally recognized by the International Code of Zoological Nomenclature (ICZN, 1999) and is defined as a population or a group of populations morphologically diagnosable that inhabit distinct breeding areas (Mayr & Ashlock, 1991)

Table 2. The COI sequence divergences determined for *Melipona (Michmelia) seminigra* complex (1–6) and the ten other *Melipona* species same subgenera (7–16), downloaded from GenBank (Ramírez et al., 2010). Sequence divergences are reported below diagonal and above are the standard deviation (SD) values; * – lowest divergence value; ** – highest divergence value.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*M. seminigra abunensis*		0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.007	0.007	0.006	0.006	0.007	0.007	0.007	0.008
2	*M. seminigra merrillae*	0.017		0.006	0.007	0.006	0.003	0.004	0.007	0.007	0.007	0.006	0.006	0.007	0.008	0.005	0.007
3	*M. seminigra* ssp1	0.020	0.015		0.008	0.005	0.007	0.006	0.007	0.007	0.007	0.006	0.006	0.007	0.008	0.007	0.008
4	*M. seminigra pernigra*	0.017	0.024	0.027		0.007	0.007	0.007	0.007	0.008	0.007	0.007	0.006	0.008	0.008	0.007	0.008
5	*M. seminigra seminigra*	0.017	0.016	0.013	0.022		0.006	0.006	0.007	0.007	0.007	0.006	0.004	0.007	0.007	0.005	0.006
6	*M. seminigra* ssp2	0.017	0.004	0.020	0.024	0.016		0.002	0.007	0.007	0.007	0.006	0.005	0.007	0.008	0.006	0.007
7	*M. seminigra atrofulva*	0.015	0.007	0.018	0.022	0.018	*0.002		0.006	0.007	0.006	0.006	0.005	0.006	0.007	0.006	0.007
8	*M. panamica*	0.024	0.022	0.024	0.024	0.020	0.022	0.020		0.002	0.003	0.006	0.005	0.007	0.007	0.006	0.005
9	*M. melanopleura*	0.026	0.024	0.027	0.027	0.022	0.024	0.022	*0.002		0.004	0.006	0.005	0.007	0.007	0.006	0.006
10	*M. costaricaensis*	0.024	0.022	0.024	0.024	0.020	0.022	0.020	0.004	0.007		0.006	0.005	0.007	0.007	0.006	0.005
11	*M. fulva*	0.020	0.018	0.015	0.022	0.016	0.018	0.015	0.015	0.018	0.015		0.004	0.005	0.006	0.005	0.006
12	*M. rufiventris*	0.017	0.015	0.018	0.018	0.009	0.015	0.013	0.011	0.013	0.011	0.009		0.005	0.006	0.005	0.005
13	*M. fuscopilosa*	0.024	0.022	0.024	0.027	0.020	0.022	0.020	0.020	0.022	0.020	0.013	0.013		0.007	0.006	0.006
14	*M. lateralis*	0.025	0.027	**0.029	**0.029	0.025	0.027	0.024	0.020	0.022	0.020	0.015	0.015	0.024		0.006	0.007
15	*M. scutellaris*	0.024	0.013	0.020	0.024	0.016	0.018	0.020	0.018	0.020	0.018	0.011	0.011	0.020	0.018		0.007
16	*M.* sp. MP85	**0.029	0.027	**0.029	**0.029	0.020	0.027	0.024	0.013	0.015	0.013	0.020	0.011	0.018	0.024	0.022	

Table 3. COI diversity of *Melipona seminigra* subspecies herein analyzed. N – number of sequenced individuals; S – number of variable sites; Hn – number of observed haplotypes; Hd – haplotype diversity; Pi – nucleotide diversity.

Subspecies	N	S	Hn	Hd	Pi	Interspecies genetic divergence (mean) %
M. s. abunensis	16	6	5	0.6670	0.0022	1.7 to 2.0 (1.76 ± 0.13)
M. s. merrillae	08	0	1	0.0000	0.0000	0.4 to 2.4 (1.58 ± 0.74)
M. s. ssp.1	09	1	2	0.2220	0.0004	1.3 to 2.7 (1.9 ± 0.54)
M. s. pernigra	15	0	1	0.0000	0.0000	1.7 to 2.7 (2.28 ± 0.37)
M. s. seminigra	14	2	3	0.2750	0.0005	1.3 to 2.2 (1.68 ± 0.32)
M. s. ssp.2	12	0	1	0.0000	0.0000	0.4 to 2.4 (1.62 ± 0.74)
Total	74	28	13			
Average				0.8770 ± 0.0140	0.0166 ± 0.0004	1.80 ± 0.47

and recently rephrased as "heritable geographic variation in phenotype" (Patten, 2015). We suggest the classification of species and subspecies in *M. seminigra* complex should be based on integrative taxonomy, as currently recommended for other animal groups (Hawlitschek et al., 2012; Torstrom et al., 2014; Patten & Remsen Jr, 2017; Winker, 2021). In the subspecies of the *M. seminigra* complex, the greatest genetic divergence was recorded for *M. s. pernigra*, ranging from 1.7 to 2.7% (average 2.28 ± 0.37) (Tables 2, 3), which is only slightly less than the among species divergence of 1.8 to 2.9% (average 2.46 ± 0.34) (Table 2). Thus, according to the COI diversity values *M. s. pernigra* seems to be close to the species threshold level, which is congruent with its phenotypic differences, mainly the black colour of the metasoma (Fig. 2c). *M. seminigra* ssp1 and *M. seminigra* ssp2 are putative subspecies (not described by taxonomists). The use of COI gene in integrative taxonomy of bees clarified taxonomic decisions, in particular the raising of *Melipona seminigra pernigra* to species level. We highlight not only the importance of *M. seminigra* as pollinator in Amazon ecosystems but also its likely derived *status* in relation to other *Melipona* species. Its chromosome number is higher than that recorded for all the other species of *Melipona* (Francini et al., 2011) and the behaviour of the workers in colonies whose queens produce diploid males differs from that expected for the genus (Francini et al., 2012). The results of this study hopefully will initiate research on the speciation and phylogeography of *M. seminigra* in the Amazon basin, which can teach us more about the domestication and conservation of this bee and its native habitat.

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Table S1. *Melipona seminigra* subspecies in which we amplified the COI gene. rmID – remnant materials ID; vID – voucher ID; COI sequences number deposited in GenBank (GenBank access). Remnant materials (preserved in absolute ethanol and stored at –80°C) and voucher specimens are deposited in the Grupo de Pesquisas em Abelhas (GPA) of the Instituto Nacional de Pesquisas da Amazônia (INPA). Note: *M. seminigra* ssp1 and *M. seminigra* ssp2 are putative new subspecies.

Subspecies	rmID	vID	Origin of sample	Date sampled	GenBank acess
M. seminigra abunensis	MS1B1	MS1B	Rondonia, Brazil	16-VI-2011	KF113947
M. seminigra abunensis	MS1B2	MS1B	Rondonia, Brazil	16-VI-2011	KF113948
M. seminigra abunensis	MS1C1	MS1C	Rondonia, Brazil	16-VI-2011	KF113949
M. seminigra abunensis	MS1C2	MS1C	Rondonia, Brazil	16-VI-2011	KF113950
M. seminigra abunensis	MS1D1	MS1D	Rondonia, Brazil	16-VI-2011	KF113951
M. seminigra abunensis	MS1D3	MS1D	Rondonia, Brazil	16-VI-2011	KF113952
M. seminigra abunensis	MS1D4	MS1D	Rondonia, Brazil	16-VI-2011	KF113953
M. seminigra abunensis	MS1E1	MS1E	Rondonia, Brazil	19-VI-2011	KF113954
M. seminigra abunensis	MS1E3	MS1E	Rondonia, Brazil	19-VI-2011	KF113955
M. seminigra abunensis	MS1F1	MS1F	Rondonia, Brazil	19-VI-2011	KF113956
M. seminigra abunensis	MS1F2	MS1F	Rondonia, Brazil	19-VI-2011	KF113957
M. seminigra abunensis	MS1G3	MS1G	Rondonia, Brazil	23-VI-2011	KF113958
M. seminigra abunensis	MS1H1	MS1H	Rondonia, Brazil	23-VI-2011	KF113959
M. seminigra abunensis	MS1H2	MS1H	Rondonia, Brazil	23-VI-2011	KF113960
M. seminigra abunensis	MS1H3	MS1H	Rondonia, Brazil	23-VI-2011	KF113961
M. seminigra abunensis	MS1H4	MS1H	Rondonia, Brazil	23-VI-2011	KF113962
M. seminigra merrillae	MS2Ca	MS2C	Amazonas, Brazil	20-XI-2007	KF113963
M. seminigra merrillae	MS2Ca2	MS2C	Amazonas, Brazil	20-XI-2007	KF113964
M. seminigra merrillae	MS2Ca6	MS2C	Amazonas, Brazil	20-XI-2007	KF113965
M. seminigra merrillae	MS2Ca7	MS2C	Amazonas, Brazil	20-XI-2007	KF113966
M. seminigra merrillae	MS2Ka1	MS2K	Amazonas, Brazil	11-IX-2008	KF113967
M. seminigra merrillae	MS2Ka2	MS2K	Amazonas, Brazil	11-IX-2008	KF113968
M. seminigra merrillae	MS2Pa1	MS2P	Amazonas, Brazil	03-XI-2008	KF113969
M. seminigra merrillae	MS2Pa2	MS2P	Amazonas, Brazil	03-XI-2008	KF113970
M. seminigra ssp1	MS2T2	MS2T	Amazonas, Brazil	20-XI-2010	KF113971
M. seminigra ssp1	MS2T6	MS2T	Amazonas, Brazil	20-XI-2010	KF113972
M. seminigra ssp1	MS2T7	MS2T	Amazonas, Brazil	20-XI-2010	KF113973
M. seminigra ssp1	MS2T9	MS2T	Amazonas, Brazil	20-XI-2010	KF113974
M. seminigra ssp1	MS2U2	MS2U	Amazonas, Brazil	20-XI-2010	KF113975
M. seminigra ssp1	MS2U11	MS2U	Amazonas, Brazil	20-XI-2010	KF113976
M. seminigra ssp1	MS2U12	MS2U	Amazonas, Brazil	20-XI-2010	KF113977
M. seminigra ssp1	MS2V1	MS2V	Amazonas, Brazil	30-XI-2010	KF113978
M. seminigra ssp1	MS2V2	MS2V	Amazonas, Brazil	30-XI-2010	KF113979
M. seminigra pernigra	MS3Aa25	MS3A	Pará, Brazil	08-VII-2010	KF113980
M. seminigra pernigra	MS3Aa26	MS3A	Pará, Brazil	08-VII-2010	KF113981
M. seminigra pernigra	MS3Ab1	MS3A	Pará, Brazil	08-VII-2010	KF113982
M. seminigra pernigra	MS3Ab2	MS3A	Pará, Brazil	08-VII-2010	KF113983
M. seminigra pernigra	MS3Ba1	MS3B	Pará, Brazil	08-VII-2010	KF113984
M. seminigra pernigra	MS3Ba2	MS3B	Pará, Brazil	08-VII-2010	KF113985
M. seminigra pernigra	MS3Bb1	MS3B	Pará, Brazil	08-VII-2010	KF113986
M. seminigra pernigra	MS3Bb2	MS3B	Pará, Brazil	08-VII-2010	KF113987
M. seminigra pernigra	MS3Ca1	MS3C	Pará, Brazil	08-VII-2010	KF113988
M. seminigra pernigra	MS3Ca2	MS3C	Pará, Brazil	08-VII-2010	KF113989
M. seminigra pernigra	MS3Ca25	MS3C	Pará, Brazil	08-VII-2010	KF113990
M. seminigra pernigra	MS3Ca26	MS3C	Pará, Brazil	08-VII-2010	KF113991
M. seminigra pernigra	MS3Cb1	MS3C	Pará, Brazil	08-VII-2010	KF113992
M. seminigra pernigra	MS3Cb2	MS3C	Pará, Brazil	08-VII-2010	KF113993
M. seminigra pernigra	MS3Da1	MS3D	Pará, Brazil	11-VII-2011	KF113994
M. seminigra seminigra	MS4A4	MS4A	Amazonas, Brazil	01-IX-2011	KF113995
M. seminigra seminigra	MS4B1	MS4B	Amazonas, Brazil	01-IX-2011	KF113996
M. seminigra seminigra	MS4B3	MS4B	Amazonas, Brazil	01-IX-2011	KF113997
M. seminigra seminigra	MS4B4	MS4B	Amazonas, Brazil	01-IX-2011	KF113998
M. seminigra seminigra	MS4D1	MS4D	Amazonas, Brazil	01-IX-2011	KF113999
M. seminigra seminigra	MS4D2	MS4D	Amazonas, Brazil	01-IX-2011	KF114000
M. seminigra seminigra	MS4E1	MS4E	Amazonas, Brazil	05-IX-2011	KF114001
M. seminigra seminigra	MS4F1	MS4F	Amazonas, Brazil	02-IX-2011	KF114002
M. seminigra seminigra	MS4G1	MS4G	Amazonas, Brazil	02-IX-2011	KF114003
M. seminigra seminigra	MS4G2	MS4G	Amazonas, Brazil	02-IX-2011	KF114004
M. seminigra seminigra	MS4G3	MS4G	Amazonas, Brazil	02-IX-2011	KF114005
M. seminigra seminigra	MS4G4	MS4G	Amazonas, Brazil	02-IX-2011	KF114006
M. seminigra seminigra	MS4H1	MS4H	Amazonas, Brazil	02-IX-2011	KF114007
M. seminigra seminigra	MS4H2	MS4H	Amazonas, Brazil	02-IX-2011	KF114008
M. seminigra ssp2	MS5A2	MS5A	Amazonas, Brazil	22-VI-2011	KF114009
M. seminigra ssp2	MS5A4	MS5A	Amazonas, Brazil	22-VI-2011	KF114010
M. seminigra ssp2	MS5B1	MS5B	Amazonas, Brazil	22-VI-2011	KF114011
M. seminigra ssp2	MS5B2	MS5B	Amazonas, Brazil	22-VI-2011	KF114012
M. seminigra ssp2	MS5C1	MS5C	Amazonas, Brazil	22-VI-2011	KF114013
M. seminigra ssp2	MS5C2	MS5C	Amazonas, Brazil	22-VI-2011	KF114014
M. seminigra ssp2	MS5D1	MS5D	Amazonas, Brazil	08-X-2011	KF114015
M. seminigra ssp2	MS5D2	MS5D	Amazonas, Brazil	08-X-2011	KF114016
M. seminigra ssp2	MS5E1	MS5E	Amazonas, Brazil	08-X-2011	KF114017
M. seminigra ssp2	MS5E2	MS5E	Amazonas, Brazil	08-X-2011	KF114018
M. seminigra ssp2	MS5F5	MS5F	Amazonas, Brazil	08-X-2011	KF114019
M. seminigra ssp2	MS5H3	MS5H	Amazonas, Brazil	10-X-2011	KF114020